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γ-Aminobutyric acid is an inhibitory neurotransmitter restricting the release of luteinizing hormone-releasing hormone before the onset of puberty

(γ-aminobutyric acid type A receptors/inhibition/hypothalamus/primates)

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ABSTRACT **To test the hypothesis that the pubertal increase in luteinizing hormone-releasing hormone (LHRH) release is withheld by a dominant inhibitory neuronal system, the role of γ-aminobutyric acid (GABA), a known inhibitory neurotransmitter, in the control of LHRH release was examined in conscious female monkeys at the prepubertal and pubertal stages using a push–pull perfusion method. GABA, bicuculline (a $GABA_A$ receptor blocker), and 2-hydroxysaclofen (a $GABA_B$ receptor blocker) were directly infused into the stalk-median eminence while perfusates were collected for LHRH determination. Bicuculline, but not saclofen, induced a large and prompt increase in LHRH release in prepubertal monkeys, whereas it stimulated LHRH release slightly in pubertal monkeys. In contrast, GABA suppressed LHRH release in pubertal, but not prepubertal, monkeys. These differential effects of GABA and the GABA antagonist on LHRH release in the two developmental stages were due to an age factor rather than to the steroid hormonal background. Moreover, GABA release in the stalk-median eminence of prepubertal monkeys was much higher than that in pubertal monkeys. Thus, the results suggest that in the prepubertal period there is a powerful GABA inhibition of the LHRH neurosecretory system: infusions of $GABA_A$, but not $GABA_B$, antagonists stimulate LHRH release by removal of the endogenous GABA inhibition, whereas exogenous GABA is ineffective because of high endogenous GABA levels. The decrease of this tonic inhibition may be a key factor for the onset of puberty in non-human primates.**

Two lines of evidence support the hypothesis that an increase in pulsatile luteinizing hormone-releasing hormone (LHRH) release is the critical factor for the onset of puberty in primates: An increase in pulsatile LHRH release occurs at the onset of puberty in female rhesus monkeys (1–3), and pulsatile infusion of LHRH into sexually immature monkeys with a pump induces precocious puberty (4). However, the mechanism by which LHRH release increases at puberty in primates is still unknown. The pubertal increase in LHRH release is probably not due to the developmental changes in properties of LHRH neurons themselves, since (*i*) the expression of LHRH mRNA is similar in monkeys during the prepubertal period and in adulthood (5) and (*ii*) prior to puberty, LHRH release can be induced by electrical stimulation of the medial basal hypothalamus (MBH) (2) or by neurochemical stimulation with *N*-methyl-D-aspartate (6). In fact, these studies further indicate that releasable LHRH is present in the hypothalamus of prepubertal monkeys but that the control mechanisms for pulsatile LHRH release are immature before the onset of puberty.

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It has been speculated for the past two decades that in primates there is a "central inhibition" of LH release, and presumably LHRH release, starting shortly after the neonatal period and ending prior to the onset of puberty (7–9). This concept is based upon the facts that, despite the absence of the gonads, LH levels in children with Turner syndrome and in neonatally castrated juvenile monkeys were as low as those in gonadally intact counterparts (10–13), and lesions in the hypothalamus resulted in precocious puberty in primates (14, 15). However, the specific mechanism of the central inhibition of LH/LHRH release is yet to be defined. If an inhibitory neurotransmitter produces tonic inhibition of the LHRH neurosecretory system, this may be implicated as a cause of central inhibition. Therefore, we searched for a possible candidate for the inhibitory neurotransmitter regulating LHRH release prior to the onset of puberty (16). Based on our earlier study† as well as on others (5), we excluded endogenous opiates in this role. Here, we have examined the role of γ-aminobutyric acid (GABA) in the control of puberty, using a push–pull perfusion method. GABA was chosen based on previous studies in adult rats (17–20).

MATERIALS AND METHODS

Animals. All female rhesus monkeys (*Macaca mulatta*) in this study were born and raised at our Primate Research Center. In experiments 1, 2, and 4, ovarian intact monkeys at the prepubertal stage (no signs of puberty evident; <20 mo of age, $n = 9$), the early pubertal stage (some signs of puberty evident, but premenarcheal; 22–27 mo of age, $n = 7$), and the midpubertal stage (postmenarcheal but before first ovulation; 34–45 mo of age, $n = 12$) were used. In experiment 3, 7 ovariectomized (OVX) monkeys at 40–60 mo of age in which other studies had been previously conducted (3, 21) were used. The time after ovariectomy was 12–24 mo. All animals were weaned at 10–11 mo of age and housed in cages (86 × 86 × 86 cm) in pairs, with 12 hr of light (0600–1800 hr) and 12 hr of darkness and at a temperature of 22°C. They were fed a standard diet of Purina Monkey Chow supplemented with fresh fruit. Tap water was available ad libitum. The protocol for this study was reviewed for the ethical use of animals in experimentation and approved by the Research Animal Resources Center, University of Wisconsin.

Push–Pull Perfusion. Before the push–pull perfusion, monkeys were implanted with a cranial chamber under halothane anesthesia (22–24). The pedestal was centered stereotaxically

Abbreviations: GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; LHRH, luteinizing hormone-releasing hormone; MBH, medial basal hypothalamus; S-ME, stalk-median eminence; OVX, ovariectomized.

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†Terasawa, E. & Chongthammakun, S., Annual Meeting of the Society for Neuroscience, Nov. 10–15, 1991, New Orleans, p. 906 (abstr.).

above the infundibular recess of the third ventricle, which was visualized with x-ray ventriculograms. The animals were allowed to recover for at least 1 mo and were well adapted to the primate chair, the experimental environment, and the investigators before experimentation was initiated (22). The method for push–pull perfusion of the stalk-median eminence (S-ME) in conscious monkeys was similar to that described previously (22–24). Three days before the push–pull perfusion, an outer cannula (20 gauge) with a inner stylet (28 gauge) was inserted stereotaxically into the S-ME with a micromanipulator unit (Narishige, Tokyo) under ketamine (10 mg/kg of body weight) plus xylazine (2 mg) anesthesia. Placement of the cannula tip into the S-ME was confirmed using x-ray pictures. The monkey was placed in a primate chair after cannula implantation and allowed to recover for 3 days before the experiment was begun. On the day of the perfusion experiment, the stylet was replaced with an inner cannula (29 gauge) and infusion was initiated. A modified Krebs–Ringer phosphate (KRP) (21) was infused through the push cannula at a rate of 23.5 μl/min and perfusates were collected on ice in 10-min fractions. Each sample was aliquoted to 150 μl for measurement of LHRH and ≈85 μl for the GABA assay. Samples were then frozen and stored at −70°C until assayed.

Experimental Design. *Experiment 1*: To determine the effects of GABA on LHRH release during pubertal development, push–pull perfusion was conducted on prepubertal (15.2 ± 0.9 mo; $n = 5$) and midpubertal (41.3 ± 2.2 mo; $n = 9$) monkeys. After 2 hr of control sampling, GABA (Sigma) dissolved in KRP at 10^{-5} or 10^{-7} M or vehicle was directly infused into the S-ME through the push cannula for 10 min at 120-min intervals for >10 hr while perfusates were continuously collected for LHRH estimation. *Experiment 2A*: To determine the role of $GABA_A$ receptors in LHRH release during pubertal development, effects of the $GABA_A$ receptor antagonist bicuculline methiodide (Sigma) were examined in prepubertal (16.2 ± 1.1 mo; $n = 6$) and midpubertal (38.8 ± 1.5 mo; $n = 6$) monkeys. After 1.5 hr of control perfusion, bicuculline dissolved in KRP at 10^{-5} or 10^{-7} M or vehicle was directly infused into the S-ME for 10 min at 90-min intervals through the push cannula while perfusates were collected. In some monkeys bicuculline at 10^{-9} M was also examined. *Experiment 2B*: To determine the role of $GABA_B$ receptors in LHRH release during pubertal development, effects of the $GABA_B$ receptor antagonist 2-hydroxysaclofen (Research Biochemicals, Natick, MA) dissolved in KRP at 10^{-5} or 10^{-7} M or vehicle were similarly examined in prepubertal (16.2 ± 0.6 mo; $n = 8$) and midpubertal (38.7 ± 1.4 mo; $n = 8$) monkeys. *Experiment 3*: To determine whether the GABA-induced LHRH suppression in midpubertal monkeys and the attenuated elevation of LHRH release by bicuculline in midpubertal monkeys are due to the increase in circulating estradiol during this stage, the effects of GABA and bicuculline were examined in OVX monkeys at mid- and late pubertal stages (49.0 ± 3.8 mo; $n = 7$). To avoid the priming effects, the order of infusion of vehicle and GABA or GABA antagonists at two concentrations was randomized. The same monkeys were assigned to multiple experiments (1–3), but none of the monkeys was used more than once in the same experiment. *Experiment 4*: GABA release in S-ME perfusates during the initial 20–90 min (before the first challenge) in 6 prepubertal (15.8 ± 1.1 mo) and 7 midpubertal (39.3 ± 2.1 mo) monkeys from experiments 1 and 2, and 7 early pubertal monkeys (25.8 ± 1.4 mo) from other experiments was determined.

LHRH Assays. LHRH in 150-μl perfusates was measured by RIA using antiserum R1245 (24). The sensitivity of the LHRH assay was 0.1 pg per tube at 95% binding. Intrassay and interassay coefficients of variation for this assay were 5.9% and 9.6%, respectively.

GABA Assay. GABA in perfusates (20 μl) was measured by HPLC with electrochemical detection (25), similar to that described previously (22). Perfusates were derivatized with a mixture of *o*-phthalaldehyde and 2-methyl-2-propanethiol in carbonate buffer (pH 9.6) and separated by centrifugation. Samples were then injected onto a C_{18} column with a mobil phase consisting of 0.05 M phosphate buffer at pH 6.6, 1 mM EDTA, 40% methanol, and 10% acetonitrile. Unconjugated *o*-phthalaldehyde and the thiol compound were eliminated with coulometric preoxidation. For an internal standard, 5-aminovaleric acid was used. Minimum detectability of GABA was 0.2 pg per sample.

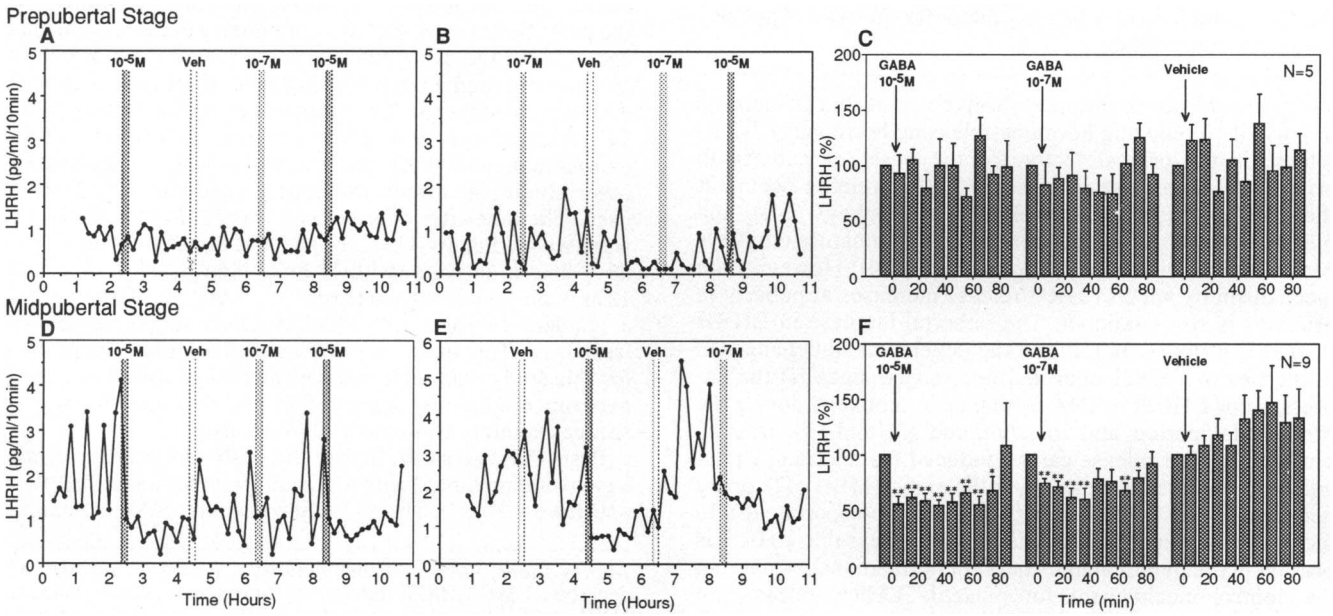

FIG. 1. Effects of direct infusion of GABA (10^{-5} or 10^{-7} M) or vehicle into the S-ME on pulsatile LHRH release in monkeys at prepubertal and midpubertal stages *in vivo*. Two representative cases (*A* and *B*) and statistical analysis (*C*) from the prepubertal group and two representative cases (*D* and *E*) and statistical analysis (*F*) from the midpubertal group are shown. GABA infusions (10^{-5} or 10^{-7} M) are indicated by the shaded bars and vehicle infusions as control are indicated by the open bars. Mean levels of LHRH release during the 20 min before the challenge infusion were designated as 100% in each animal. **, $P < 0.01$; *, $P < 0.05$ vs. mean LHRH release 0–20 min before the infusion.

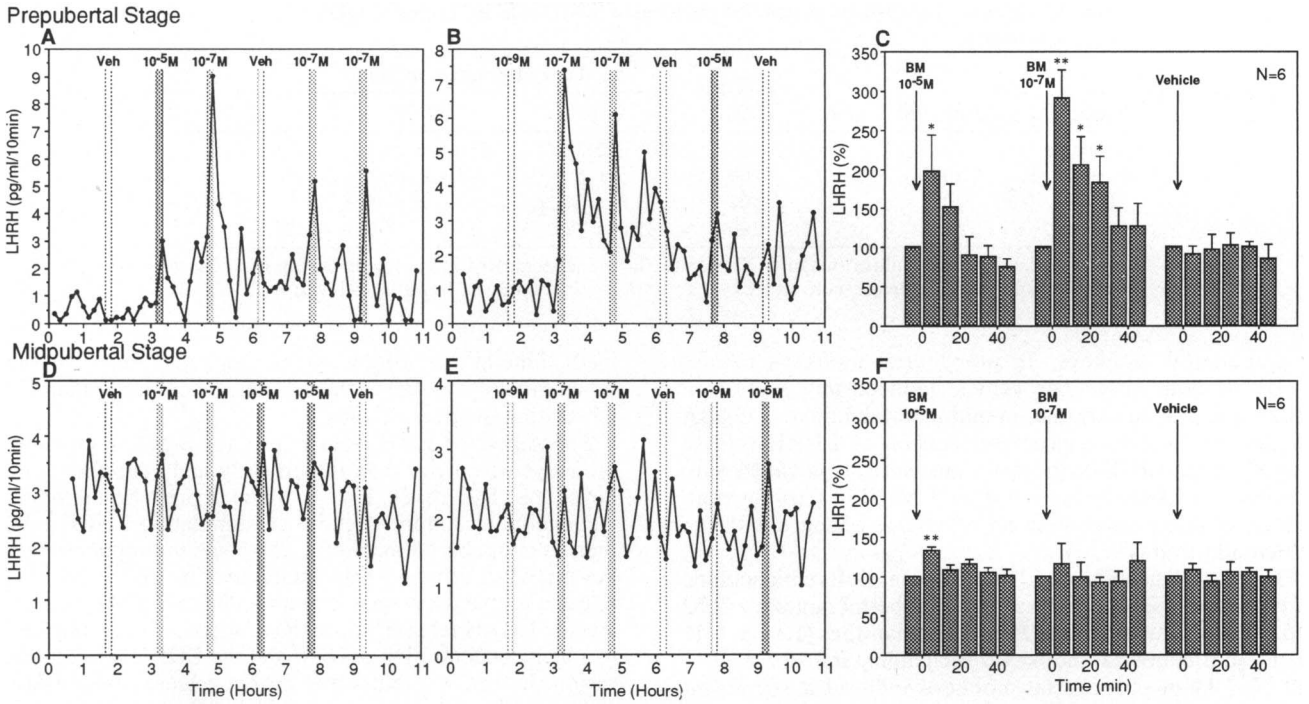

FIG. 2. Effects of direct infusion of bicuculline (BM: 10^{-5} or 10^{-7} M) or vehicle into the S-ME on pulsatile LHRH release in monkeys at prepubertal and midpubertal stages *in vivo*. Two representative cases (*A* and *B*) and statistical analysis (*C*) from the prepubertal group and two representative cases (*D* and *E*) and statistical analysis (*F*) from the midpubertal group are shown. Bicuculline infusions (10^{-5} or 10^{-7} M) are indicated by the shaded bars and vehicle infusions as control are indicated by the open bars. The mean LHRH release during the 20 min before the infusion was expressed as 100%. **, $P < 0.01$; *, $P < 0.05$ vs. mean LHRH release 0–20 min before the infusion.

Statistical Analysis. Developmental changes in GABA and LHRH release and the effects of GABA or GABA antagonists were determined by using analysis of variance with repeated measurements followed by post hoc Student–Newman–Keuls multiple range tests. Mean levels of LHRH during the 20-min period before each infusion were compared to the LHRH levels in samples from the following successive 10-min periods. The results from more than one challenge within an experiment were averaged to make a single entry per animal for statistical analysis. Since there were large individual variations within a group, data were analyzed after the $\log x + 1$ transformation (1). Significance was attained at $P < 0.05$. For graphic expression, data were normalized: the mean LHRH levels during the 20-min period before each challenge infusion were expressed as 100% and the values after the infusions were calculated accordingly.

RESULTS

Effects of GABA on LHRH Release. Before GABA infusion, mean LHRH release (3.57 ± 0.65 pg/ml) in midpubertal monkeys was higher ($P < 0.01$) than that (1.23 ± 0.3 pg/ml)

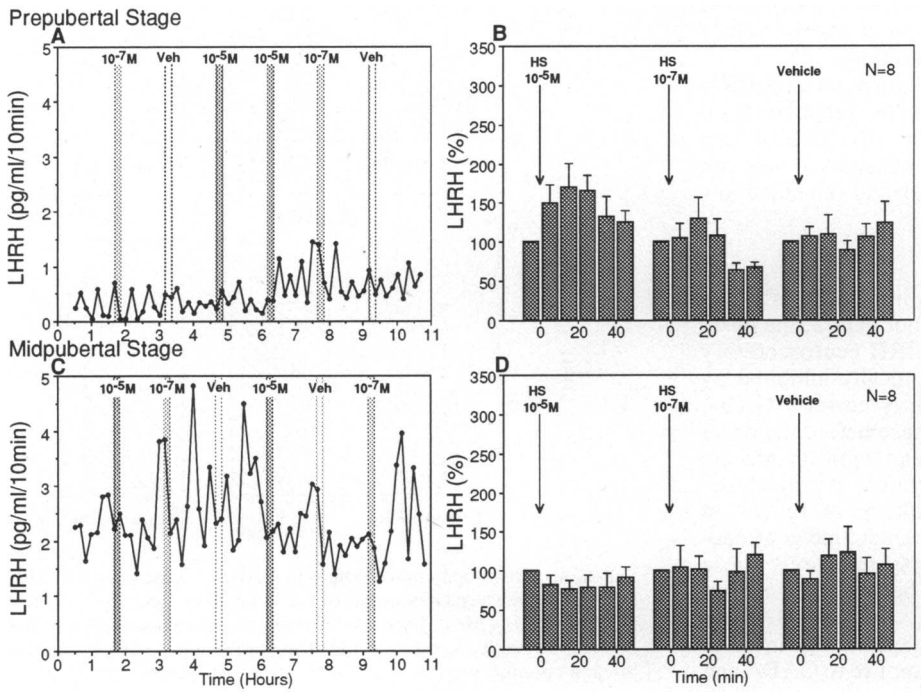

FIG. 3. Effects of direct infusion of saclofen (HS: 10^{-5} or 10^{-7} M) or vehicle into the S-ME on pulsatile LHRH release in monkeys at prepubertal and midpubertal stages *in vivo*. Examples of a prepubertal monkey (*A*) and a midpubertal monkey (*C*) are shown. Saclofen infusions (10^{-5} or 10^{-7} M) are indicated by the shaded bars and vehicle infusions as control are indicated by the open bars. Statistical analyses for prepubertal monkeys (*B*) and for midpubertal monkeys (*D*) are also shown.

Table 1. Effects of GABA or bicuculline methiodide (BM) on LHRH release in OVX pubertal monkeys

Treatment	Conc., M	No.	Time after infusion, min			
			−20–0	0–10	10–20	20–30
Vehicle		7	100	105.0 ± 22.7	110.6 ± 20.2	138.6 ± 35.1
GABA	10^{-5}	5	100	48.5 ± 6.1**	48.4 ± 6.1**	50.8 ± 10.0*
BM	10^{-5}	4	100	88.8 ± 24.1	95.6 ± 30.5	76.6 ± 24.0
BM	10^{-7}	7	100	135.0 ± 38.6	85.9 ± 11.9	127.1 ± 41.1

Normalized LHRH values (mean ± SEM) are shown. The mean LHRH levels during the 20-min period before each challenge were expressed as 100%. *, $P < 0.03$ vs. preinfusion level; **, $P < 0.01$ vs. preinfusion level.

in prepubertal monkeys. In prepubertal monkeys neither GABA at both doses nor vehicle induced any significant effects (Fig. 1). In contrast, in midpubertal monkeys GABA infusion resulted in a clear suppression of LHRH release (Fig. 1): mean LHRH concentrations were significantly suppressed by GABA at 10^{-5} and 10^{-7} M ($P < 0.01$ for both doses), whereas there were no significant changes with the vehicle infusion (Fig. 1).

Effects of Bicuculline on LHRH Release. Before bicuculline infusion, mean LHRH release in midpubertal monkeys (2.62 ± 0.64 pg/ml) was higher ($P < 0.01$) than that (1.14 ± 0.16 pg/ml) in prepubertal monkeys. Bicuculline infusion at 10^{-5} and 10^{-7} M in prepubertal monkeys induced a significant increase in LHRH release ($P < 0.03$ for 10^{-5} M; $P < 0.01$ for 10^{-7} M, Fig. 2), whereas vehicle infusion did not cause any effects. In contrast, in midpubertal monkeys only bicuculline at 10^{-5} M was effective in increasing LHRH release (Fig. 2).

Effects of Saclofen on LHRH Release. Saclofen did not cause any consistent results in either prepubertal or midpubertal monkeys (Fig. 3). Similarly, vehicle failed to cause any effects on LHRH release (Fig. 3).

OVX Monkeys. GABA infusion at 10^{-5} M in OVX pubertal monkeys ($n = 7$) decreased LHRH release significantly ($P < 0.01$, Table 1). The degree of the suppression in OVX monkeys was similar to that in ovarian intact midpubertal monkeys. However, bicuculline at 10^{-5} and 10^{-7} M did not cause any significant effects in OVX monkeys (Table 1).

Behavioral Changes. Because the neurotransmitter GABA is known to be involved in many brain functions, we carefully observed the animals for behavioral changes during and after the infusion of GABA or its antagonists. GABA, bicuculline, and saclofen did not cause any behavioral changes, such as seizures, or changes in food intake or drinking.

GABA Release During Puberty. Although mean LHRH release progressively increased during the prepubertal to early pubertal and midpubertal stages (Fig. 4*B*), GABA levels measured in the same samples drastically decreased between the prepubertal and early pubertal stages and remained low during the midpubertal stage (Fig. 4*A*).

DISCUSSION

The finding that direct GABA infusion into the S-ME suppresses the release of LHRH after, but not before, the onset of puberty led to the hypothesis that LHRH neurosecretory neurons in prepubertal monkeys are tonically inhibited by endogenous GABA. If this hypothesis is correct, GABA antagonists should stimulate LHRH release before the onset of puberty. Indeed, the results of GABA antagonists indicate that infusion of bicuculline, but not saclofen, clearly stimulates LHRH release in prepubertal monkeys. Moreover, in midpubertal monkeys the response to bicuculline is attenuated when compared to that of prepubertal monkeys. These results suggest that GABA inhibition before the onset of puberty is mediated through $GABA_A$, but not $GABA_B$, receptors. Moreover, the results from experiments in OVX monkeys indicate that the differential response to GABA and bicuculline in the two developmental stages seen in ovarian intact monkeys is due to the age difference rather than to the circulating ovarian steroids.

Spontaneous LHRH release from the S-ME in prepubertal monkeys was lower than that in early and midpubertal monkeys (refs. 1–3, 16, 26; Fig. 4). Since in prepubertal monkeys bicuculline was more effective in stimulating LHRH release than in midpubertal monkeys, and since in prepubertal monkeys GABA infusion was ineffective in inhibiting LHRH release, unlike the response in midpubertal monkeys, the low level of LHRH release before the onset of puberty may be due to a high concentration of GABA at the LHRH neuroterminal region. Indeed, we found that GABA release in the S-ME of prepubertal monkeys was much higher than that of early and midpubertal monkeys. Therefore, it appears that elevated GABA release at LHRH neuroterminals is responsible for the prepubertal inhibition of LHRH release.

Whether inhibitory GABA action on LHRH release occurs directly on LHRH neurons or indirectly through interneurons is unknown. In rats, synaptic inputs of glutamic acid decarboxylase (GAD)-positive neurons were found on LHRH-positive perikarya located in the preoptic area (27). In the rhesus monkey, many LHRH neurons are distributed in the MBH, supraoptic nucleus, and medial preoptic area (28, 29),

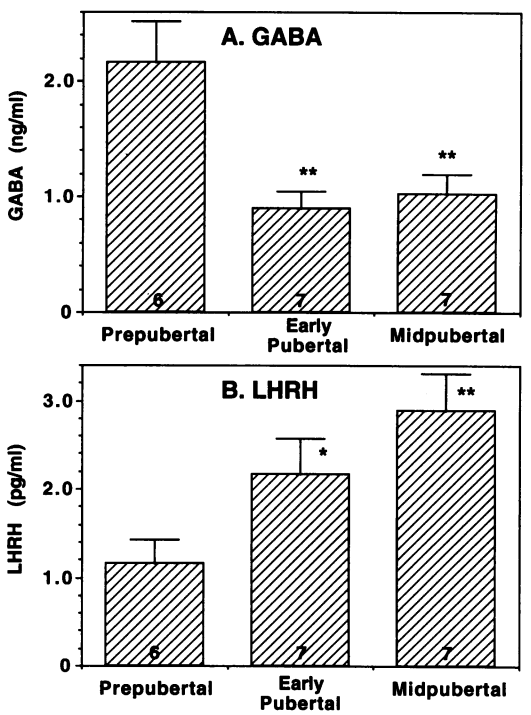

FIG. 4. Developmental changes in GABA release (*A*) and LHRH release (*B*) measured in aliquots of the same 10-min perfusate samples from the S-ME. Mean (±SEM) release was calculated from averages of several samples in each animal. The number of animals is indicated in each column. **, $P < 0.01$; *, $P < 0.05$ vs. prepubertal stage.

and GAD-positive perikarya are present in the arcuate nucleus and dorsomedial hypothalamus (30, 31). Although no direct synapses of GAD-positive neurons onto LHRH perikarya were observed in the supraoptic nucleus,‡ it is not known whether GABA neurons synapse on LHRH neurons in the MBH.

It is possible that GABA and bicuculline exert their effects on LHRH release by acting directly on the neuroterminals of LHRH neurons in the S-ME. Infusion of these neuroactive substances and simultaneous samplings are from a restricted area within the S-ME (32), where abundant LHRH neuroterminals but only a small number of LHRH perikarya are present (28, 29). In fact, a recent report indicating that GABA blocked action potentials through the $GABA_A$ receptor–Cl^- channel complex at the neuroterminals of oxytocin/vasopressin neurons (33) suggests that the neuroterminal is an important site of neurotransmitter action for the control of neurosecretion. Whether there is any direct contact between the LHRH and GABA neuroterminals remains to be investigated.

At least three mechanisms for the reduction in endogenous GABA release leading to the increase in LHRH release and the subsequent initiation of puberty can be speculated: (*i*) morphologically, GABAergic synapses onto LHRH perikarya and/or neuroterminals may be reduced as a programmed developmental event; (*ii*) biochemically, synthesis of GABA may be reduced due to developmental changes in the biosynthetic enzyme, GAD; or (*iii*) physiologically, neural activity of GABAergic neurons may be reduced during the development of the hypothalamus. Specific mechanisms are presently unknown.

Since in prepubertal monkeys, high levels of GABA in the area enriched by LHRH neuroterminals appear to be the cause of low levels of LHRH release, we have speculated that an inhibition of GAD synthesis that presumably reduces GABA synthesis and subsequent GABA release in prepubertal monkeys may alter the LHRH release. In a preliminary study§ we found that infusion of an antisense oligonucleotide (20-mer) for GAD67 mRNA directly into the S-ME of prepubertal monkeys resulted in a drastic increase in LHRH release, whereas treatment with an oligodeoxynucleotide containing the same bases in a scrambled sequence failed to induce any significant change. Infusing an antisense oligodeoxynucleotide for GAD65 mRNA also induced an increase in LHRH release but of a lesser magnitude. The antisense oligodeoxynucleotides were presumably taken into the cell blocking GAD synthesis. Although GABA levels after the antisense DNA treatment are yet to be determined, these findings are consistent with the present study in which removal of GABA inhibition increases LHRH release.

Is the removal of GABA inhibition enough to bring about full puberty? Since we have found that excitatory inputs from norepinephrine and neuropeptide Y neurons to LHRH neurons are contributing factors for the pubertal increase in LHRH release (26, 34), and since others have shown that stimulation of *N*-methyl-D-aspartate receptors results in precocious puberty (35, 36), the establishment of excitatory neuronal inputs to the LHRH neurosecretory system may occur subsequently or concurrently with the removal of GABA inhibition prior to the initiation of puberty. Further detailed studies are required to clarify the regulation of LHRH release by other neurotransmitters during puberty.

In summary, we have tested the hypothesis that GABA plays a role in the tonic inhibition of LHRH release before the onset of puberty in female rhesus monkeys. We have found that low levels of LHRH release in the prepubertal period appear to be due to the dominant inhibitory mechanism of GABA mediated by the $GABA_A$ receptor and that the removal of this inhibition may trigger an increase in LHRH release and the onset of puberty.

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